Circadian Clocks: 50 Years On

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Since the first Cold Spring Harbor meeting on "Biological Clocks" in 1960, the field has progressed from the study of a fascinating but esoteric set of phenomena of interest primarily to a relatively small group of prescient biologists to become recognized as defining a centrally important aspect of biological organization. This change is the consequence of a profound increase in understanding of the mechanisms that generate and control circadian rhythmicity, coupled with the realization that circadian temporal organization is an important component of much of what most organisms do. As such, it impinges on human health, agriculture, and biological conservation, as well as on many more basic aspects of biology at every level. Many of the seminal discoveries of the last 47 years were presented and discussed at this exciting meeting.

Well, almost 50 years have gone by—the 25th Cold Spring Harbor Symposium on Quantitative Biology "Biological Clocks" was held in June of 1960. The 72nd, only the second devoted to this subject, followed it by 47 years. During those years, our understanding of circadian phenomena has grown exponentially, in parallel with much of the rest of biology. Although we now know a great deal more than we did in 1960, the seeds of our current understanding are present in volume 25, and it is instructive both scientifically and historically to ask what caused them to germinate, to grow, and, in some cases, to flower.

Most importantly, they were very good seeds. Although what we knew then was almost exclusively phenomenological, the phenomena were extraordinarily interesting. Circadian timekeeping was precise, in some cases almost unbelievably so; Pat DeCoursey's flying squirrels began their nightly activity with an accuracy of a few minutes, about 1 part in 30,000, in the complete absence of external time referents. Other overt rhythms were almost as good. Such precision demands a highly evolved control system that, almost by definition, can be unraveled when appropriate techniques are available. Although not available in 1960, such techniques were already on the way.

Circadian rhythmicity—with very similar formal properties—was found in nearly all organisms: protists, fungi, plants, and a variety of animal species. Once you leave the realm of cells and subcellular organization, that level of generality is rare in biology and can only mean that the phenomenon is of fundamental importance. Several different protists *(Euglena, Gonyaulax)* had circadian rhythms, demonstrating that the requisite machinery could be packed into a single cell.

The fact that the circadian period was close to a day, could be synchronized by environmental cues to exactly 24 hours, and, importantly, to a determinate phase relationship with those cues, suggested that the mechanism could function as a clock. This suggestion was supported by some dramatic examples, in particular, sun compass orientation, which could be manipulated by manipulating circadian timing. The idea of a biological clock made of cells, and ultimately, of molecules, caught people's imaginations and led Pittendrigh to search for and then clearly demonstrate the unusual property of temperature-compensated period.

Finally, it was clear that a great deal of biochemistry, physiology, and behavior was rhythmic with circadian periodicity, confirming the fundamental importance of the phenomenon, underlining its potential adaptive significance, and suggesting its possible involvement in various pathologies. The importance of these possibilities was brought home by the demonstration that human beings had circadian rhythms that were indistinguishable from those of other mammals.

In 1960, the study of biological clocks was at the same point in its logical development as was the study of genetics 60 years earlier, before the chromosome theory of heredity. Clocks, like "heredity factors," were locked in a black box that could be studied only by manipulating its outputs. One could study the results of crosses in the one case and light pulses in the other, deriving in both cases information that would become a vital foundation for subsequent analysis, but what was inside the black box was completely unknown. Not only its contents, but even its location was a mystery and in neither case could the box be unlocked until it was found. Because of the demonstration that hereditary factors were located on chromosomes, the "chromosome theory" quickly generated a large body of new and exciting work; for circadian rhythms, the path to the core oscillator was longer and more convoluted.

Tremendous progress has been made since 1960. Most obviously, there has been an explosive increase in our understanding of circadian mechanisms at several levels of organization. This began with the identification of circadian pacemakers in multicellular organisms: silk moths, cockroaches, *Aplysia*, birds, and mammals. It is still ongoing in *Drosophila*, where painstaking neuroanatomy combined with genetics and behavior is revealing the circadian function of specific neurons in the brain

All authors cited here without dates refer to papers in this volume.

(Helfrich-Förster et al.), and in mammals, where inputs to and outputs from the suprachiasmatic nucleus (SCN) are being mapped and their functions pursued (Güler et al.; Doyle and Menaker; Yan et al.; Saper and Fuller). There is still much of importance to learn at this level of organization, especially in mammals; the location of circadian oscillators that respond to food and drugs such as methamphetamine is unknown, and output pathways that connect central pacemakers with peripheral organs and rhythmic behaviors such as sleep are under intense investigation. Work at this level is likely to yield medically important insights as the relationship of circadian rhythmicity to a large range of normal physiological processes and to many specific pathologies becomes more widely appreciated.

The initial identification of pacemaking structures preceded the genetic approaches pioneered in *Drosophila* by Konopka and Benzer (1971) and in *Neurospora* by Dunlap and Feldman (1988). For a while, genetics and functional anatomy proceeded along parallel paths, but when genetic insights began to produce molecular tools, the paths merged. This merger has recently produced a cornucopia of new data that has enabled a rapid increase in our understanding of fundamental circadian mechanisms. Summaries of that increase form a large portion of the content of this volume.

Outlines of the central molecular loops that generate circadian oscillations have been worked out during the past several years for flies, mice, *Neurospora*, *Arabidopsis,* and the cyanobacterium *Synechococcus elongatus.* The outline is most complete for *Synechococcus*, least complete for *Arabidopsis.* At the 1960 meeting, there was much speculation about the possible existence of circadian rhythms in bacteria and discussion of the great analytical advantages of a bacterial clock system if one could be found. It took quite a while, but when circadian rhythmicity was discovered in *Synechococcus*, the predicted analytical progress came rapidly. Current understanding of the mechanism of the circadian oscillator in *Synechococcus* has become the gold standard to which students committed to the reductive analysis of other circadian systems aspire. The circadian cycle can be generated in vitro by incubating three bacterial proteins with ATP. The "artificial" rhythm is temperature-compensated and its period matches that of wild-type mutant strains of the bacteria from which the proteins are derived (Kondo; Johnson). Since the Cold Spring Harbor Laboratory meeting took place, a new paper has appeared proposing a detailed quantitative model of the phosphorylation events that drive the oscillation (Rust et al. 2007).

These results have settled several 1960 questions. Obviously, bacteria, although probably a very limited subset, can have circadian rhythms with all their essential properties. Circadian rhythmicity is likely to be very very old because what we know about cyanobacteria suggests that they have not changed much in the last 2–3 billion years. Because there are no mechanistic homologies between clocks in cyanobacteria and those in other groups of organisms, they have almost certainly evolved independently. Indeed, what we know about clock mechanisms in general suggests at least three independent origins in cyanobacteria, plants, and animals and possibly four, depending on how one feels about the fungi. If that is true, convergence at the formal level, i.e., temperature-compensation, response to light, range of period, has been remarkable.

Although timekeeping in cyanobacteria does not appear to require a transcription-translation feedback loop, virtually all transcription in this organism is regulated in a circadian manner. Fascinating questions remain concerning the ways in which the protein clock regulates gene expression and other cellular activities (Golden). Circadian timekeeping in other organisms certainly involves transcription-translation mechanisms, but are they absolutely essential? Experiments in flies suggest that they probably are (Rosbash et al.), but the existence of protein clocks in complex organisms cannot be excluded.

A group of papers addresses our still incomplete understanding of the circadian-rhythm-generating mechanisms in eukaryotes. Clearly, we have not yet identified many of the genes that regulate circadian rhythmicity in mammals, as there are as many as 20 or more mutant phenotypes with unknown genetic bases (Siepka et al.). In *Drosophila*, studies of the properties of gene networks suggest that many genes that do not show up in mutant screens have important effects on circadian phenotype and that genes involved in the clock mechanism can be greatly influenced by many other genes (Foltenyi et al.; Hall et al.). Among these may be clock-controlled genes that regulate processes that evade particular screens but may feed back on core oscillators and modify their properties. Gene-chip analysis of clock neurons has been used to identify candidate genes of this kind (Blau et al.).

Unraveling the mechanisms that extend the time required to complete a circadian loop remains a high priority. Not surprisingly, as in cyanobacteria, phosphorylation is centrally important, probably in all such systems. It is an important regulator of circadian period and is responsible for some of the built-in delays that produce near 24-hour cycles from biochemical oscillations that would otherwise run much faster (Virshup et al.; Querfurth et al.; Vanselow and Kramer; Maywood et al.). It may also be involved in the still mysterious mechanism of temperature-compensation of period length (Dunlap et al.). Other period-extending mechanisms under study include the incorporation of fixed interval timers within the circadian cycle (Saez et al.) and chromatin remodeling by modification of histones (Grimaldi et al.).

Posttranscriptional mechanisms act both within the core circadian loop and on target mRNAs to regulate rhythmic expression patterns and thus clock outputs. A variety of such mechanisms have major effects on the period of the circadian cycle (Vanselow and Kramer) and on its maintenance (Somers et al.). Posttranscriptional mechanisms also have a significant role in shaping the circadian profiles of clock-controlled genes (Garbarino-Pico and Green; Keene) and are thus important and, until recently, underappreciated components of the output pathways that link the central circadian loop to many critical aspects of physiology and behavior (Foltenyi et al.; Chen et al.; Loros et al.; de Paula et al.). Degradation of

cryptochromes (and probably other clock proteins) is involved in regulating both period and amplitude of circadian rhythms in mice (M. Pagano, unpubl.; Siepka et al.; Maywood et al.).

During the past 10 years, it has become clear that clocks are widely distributed *within* multicellular organisms. This was anticipated in 1960 because of the many different rhythms that could be observed in an individual and the presence of clocks in single-celled protists. However, the unequivocal demonstration that there were self-sustained oscillators with a full range of circadian properties in the cells, tissues, and organs of eukaryotes awaited the advent of dynamic molecular reporters, chiefly luciferase. These, when combined with transgenic and transient transfection technology, made it possible to satisfy the basic condition for efficient circadian experimentation: long-term automatic recording of rhythmicity. Cells and tissues from transgenic animals with luciferase reporting the circadian transcription of clock genes were cultured and rhythms of light output measured with photomultipliers. Most displayed rhythmicity in vitro with varying degrees of robustness. These results brought home to the field, in ways that no amount of inference could, that we were dealing not with a single molecular or neural oscillator, not with a single measured behavior, but with a complex system. As students of biological organization, we have to understand its system properties as well as the properties of its individual components.

Complete analysis of any biological system involves at least five steps: identifying its components, discovering their individual properties, understanding the links among them and their interactions with each other, learning how the system responds to the environment, and, finally, how it functions adaptively in nature. This is clearly a daunting task and one which, for "the" circadian system, has barely begun. It is important to recognize that despite the significant molecular homologies among the cell-autonomous circadian oscillators of eukaryotes, many important system-level details will vary widely among species. For mammals, the currently available tools dictate an emphasis on mice and, to a lesser extent, on rats, but while acknowledging their advantages, we should also be aware of their limitations, of which two are major. First, these laboratory rodents are no longer real animals and so it is almost meaningless to ask how their circadian systems function adaptively in nature. The second limitation is particularly important in light of the use of these animals as models of human disease. It is clear that that aspect of circadian research promises new and important insights into a wide variety of pathologies (see more below). For many aspects of mammalian physiology, rats and mice are reasonable first approximations of humans, but for studies aimed at identifying circadian influences, an important distinction between these rodent models and humans must be kept in mind: We are diurnal, and the rodents are strongly nocturnal. Because circadian systems evolve under strong selective pressure to maximize the adaptive significance of phase control, this distinction is likely to have important consequences. These may crop up not only in circadian studies, but also as aspects of classical homeostatic physiology are examined at greater depth. We cannot abandon these models, but we do need to make comparisons with the admittedly less convenient diurnal models where possible.

Several contributions to the present volume report real progress in exploring the links between central and peripheral clocks. Such links may well involve tissue-specific nuclear receptors which are shown to oscillate with circadian periods and could function as part of a network coupling circadian clock outputs to metabolism, to reproduction, and, secondarily, to hormonally regulated behavior (Yang et al.). Circadian oscillators in peripheral organs regulate the expression of large numbers of so-called clock-controlled genes (CCGs). Most of the genes so regulated are tissue-specific; for example, of the more than 300 CCGs in heart and liver, only about 10% are common to both organs (Storch et al. 2002). This strongly suggests that each organ has its own functionally significant circadian gene expression profile, although it has been difficult to connect the details of such gene expression with organ-specific functions. Experiments with transgenic mice engineered to conditionally ablate clock function in the liver alone or in the entire animal with the exception of the liver have led to the interesting conclusion that cyclic expression of most (90%) liver CCGs depends on a functional clock in the liver itself (Kornmann et al.). These results leave open the question of whether the liver clock directly drives circadian gene expression or interprets signals from the SCN or elsewhere. Without a liver clock mice fail to regulate glucose levels normally. When clock function is limited to the liver alone, its cells can still be synchronized to daily cycles of food availability (Storch et al.). These results suggest that the liver operates with more independence from central oscillators than might be expected in a highly integrated system. That in itself might be an advantage in a world in which food availability may be quasirhythmic and not always phase-locked to the day/night cycle. The ability of a liver clock to respond flexibly to changing rhythms of food availability could be particularly useful to "weed" species like rats and mice. It will be interesting to see if similar patterns of control operate in other tissues and in the livers of organisms that have rigidly timed feeding opportunities in nature.

If, as seems almost certain, circadian clocks pervasively regulate important aspects of cell and organ function, it would not be surprising to discover that they are involved in a wide range of pathologies. Hints of such involvement come from several sources. The deleterious effects of time shifts, be they the result of jet lag or shift work, are well known to most people from personal experience, although in most of the scientifically controlled studies, it has not been possible to separate effects on the integrity of the circadian system or on rhythmicity of specific functions from the effects of fatigue produced by sleep disruption.

Rhythmic sleep may be simply an output of the circadian system, such as rhythmic body temperature, or its interaction with central circadian oscillators may be more intimate (M. Yanagisawa, unpubl.; Saper and Fuller; Tafti and Franken). Sleep deprivation is such a drastic treatment for most mammals that it is difficult to untangle its many

effects. One way to study the interaction of sleep and circadian rhythms is to use model organisms that normally have consolidated sleep but seem able to do without it. Flies (Sehgal et al.) and zebra fish may fall into this category, but with a few exceptions, the field has neglected a promising model in Passerine birds that appear perfectly healthy after months without consolidated sleep (Gaston and Menaker 1968; Rattenborg et al. 2004).

Mutations in genes that are part of the circadian rhythm-generating loop have major effects not only on molecular events in the loop, but also on a variety of other processes. Some of these are clearly related to the circadian function of the gene involved. Perhaps the best examples are the mutations in humans that cause familial advanced sleep phase syndrome (FASPS) (Ptáček et al.) and their orthologs in model organisms (Loudon et al.). Such mutations are especially useful because they open the connections between molecular and physiological processes to further analysis. Other more general effects of circadian mutations are more difficult to interpret because it is usually unclear whether the effect of the mutation is only or even primarily on the circadian system or on some noncircadian function of the gene. In this category are circadian mutations that produce phenotypes that may mimic psychiatric disorders of humans (McClung) as well as reproductive disorders, bone and muscle defects, and cancer (Gery and Koeffler). Although these phenotypes are difficult to ascribe to particular clock-related mechanisms, their existence underlines the wide influence that this system, taken in its broadest sense, exerts on normal function.

A different health-related aspect of circadian organization derives from the fact, clear already in 1960, that there are robust circadian rhythms of sensitivity to a variety of environmental insults (Kondratov and Antoch). Efforts to take advantage of such rhythms for therapeutic purposes, e.g., chronotherapy for cancer treatment, are showing promising results (Lévi et al.) and should improve as we learn more about the ways in which the circadian system interacts with many aspects of basic physiology and, in particular, with the cell cycle.

Modeling at some level is implicit in every scientific undertaking, and explicit modeling of circadian organization has been an important aspect of the field since its inception. Many of the early models rested on analogies with physical oscillators and were particularly useful in suggesting experiments designed to explore how far those analogies could be pushed, e.g., phase-response curves, limits of entrainment, aftereffects, and frequency demultiplication. Now that so much more is known about detailed circadian mechanisms, modelers are faced with the task of incorporating into their models what is known about the interactions among multiple negative feedback loops involving many genes and proteins. Such models have been developed for plant (Millar et al.) and mammalian circadian systems (Ueda). They are useful for organizing large bodies of data and inferring logical structure, but the challenge is to use them to predict unanticipated system properties or components. Some success has already been achieved. Another approach to understanding the basic logic of oscillating networks is to compare several and attempt to extract common features that are essential to their function. This comparative approach has been a staple of biological analysis for hundreds of years, but it is now possible to apply it at the fundamental molecular level. It has the potential to provide important insights into the structure of both circadian (Mockler et al.) and higher-frequency metabolic cycles (Tu and McKnight; Hughes et al.).

From a comparative point of view, new biological models are always welcome. Work with lepidopteran species and other insects suggests that *Drosophila* may be atypical in having only one *Cry* gene. The differences between the molecular aspects of circadian organization in *Drosophila* and the monarch butterfly (Reppert) suggest that the *Drosophila* pattern may be highly derived and point to the dangers of inferring evolutionary history from a small number of model species chosen for convenience.

Implied comparison on yet another level is exemplified by a group of papers dealing with noncircadian aspects of temporal organization. These deal with the "clock" that underlies the development of body segmentation (Kageyama et al.; Pourquié) and with aging (C. Kenyon, unpubl.; Guarente; Ruvkun et al.). Although there is as yet no evidence of a direct relationship between these processes and circadian rhythmicity, it is not out of the question that some of the same genes may be involved in their control. There is at least one good example of that kind of pleiotropy in the regulation by the *Drosophila Per* gene of the period length of both the circadian rhythm and the much higher-frequency rhythm of wing vibration used by courting male flies (Konopka et al. 1996). Other circadian genes are expressed during development of *Drosophila* in both oscillator cell precursors and nonoscillator cells; however, their function in these latter cells is unknown (Benito et al.).

The importance of timing to events in development was beautifully underlined by Martin Raff in the Reginald B. Harris Lecture. He described in vitro experiments with oligodendrocyte precursor cells which contain an internal timer that schedules cessation of cell division and initiation of differentiation (Raff). The timing in vitro parallels the timing in vivo and depends in part on the levels of two proteins. Control is at both the transcriptional and post-transcriptional levels.

The circadian rhythms of human beings are very much the same as those of other mammals. Their responses to the physical environment (chiefly photic) are similar to those of other diurnal mammals. However, the interaction of circadian mechanisms with the social environment produces unique behavioral and physiological responses. Furthermore, the social environment produces major modifications of the photic environment, in particular, extension of the photoperiod by artificial light and concomitant reduction in overall light intensity as a consequence of indoor living. The obvious disadvantages of humans as experimental subjects are at least partially offset by some unique advantages. Humans are more cooperative than mice. They sit still, answer questions, fill out questionnaires, spit in tubes, and urinate in cups on command, and they have lots of blood. Even though breeding experiments are out, there are many natural experiments

going on all the time; the breeding population is very large and variable, it is found in geographically diverse locations, and its genome is known in great detail. Some of these advantages have been exploited in circadian studies. The distribution of phases of sleep and activity rhythms in a very large sample has been determined by using a simple questionnaire (Roenneberg and Merrow). The large sample size enables a useful descriptive analysis of human chronotype by age, sex, occupation, etc. At the extremes of an almost normal distribution are "larks" and "owls" waiting for genetic/molecular analysis which has already been successfully initiated in the study of FASPS (Ptáček et al.).

Compared to its central role in the circadian systems of many nonmammalian vertebrates (Menaker and Tosini 1996) and in the control of reproduction in photoperiodically regulated, seasonally breeding mammals (Goldman 2001), the role of melatonin in the physiology of mammalian species who do not breed seasonally is disappointingly minor. Melatonin has small effects on the entrainment of mammalian behavioral rhythms which are nonetheless useful clinically in helping blind humans to synchronize to their environment, but its most significant use is as a reliable marker of circadian phase (Lewy). It may be involved at some level in psychiatric disorders such as depression and autism (Bourgeron), perhaps in memory formation (Rawashdeh et al. 2007), and in some cancers, but it is hard to escape the feeling that despite a great deal of work, we have still not identified its basic function in mammals.

The importance of understanding the detailed interaction of the human circadian system with the social and physical environment that we have created for ourselves was made dramatically clear in the Dorcas Cumming Lecture presented by Charles Czeisler. Using hard data collected primarily from doctors at stages in their careers at which they were required to work long noncircadian schedules, he described their involvement in driving mishaps and potential for medical mistakes (Czeisler et al.). As fatigue increases, judgment declines progressively, so that severely fatigued individuals, like people who have had too much to drink, do not realize that they are impaired. Understanding of the social costs of fatigue is one of the most important practical benefits that could be derived from our current knowledge of the circadian regulation of sleep. It is frustrating to see it ignored by those who design work schedules for pilots, truck drivers, shift workers, and medical residents.

Circadian rhythmicity is one of the most obvious and easily studied aspects of the much broader problem of understanding the temporal organization of living systems. The temporal program that underlies biological clocks is particularly amenable to analysis, and a mere 50 years of work has revealed a great many of its secrets—at an unprecedented array of organization levels from behavior to molecular structure. This may prove to be a model for future work on the temporal structure of other biological systems; at the least, it cannot help but draw attention to the importance of time in biology.

The field of biological clocks has always been exceptionally broad both in terms of the model systems studied and in the endpoints measured. It has often seemed on the verge of subdividing along either organism or process lines, but it has been repeatedly rescued by appreciation of the deep formal similarities among its subjects. Its breadth has made it a unique meeting ground for scientists with very different backgrounds and goals. The tendency to draw people in from other fields with new approaches and fresh ideas has contributed in a major way to its rapid growth. That tendency is likely to accelerate as the multiple dynamic connections between circadian temporal programs and other aspects of biological organization become more widely recognized. This will have important practical consequences for medicine, for agriculture, and for species conservation. It would be a shame if the field continued its neglect of its defining but admittedly most difficult question: How do animals, plants, fungi, and bacteria make adaptive use of their biological clocks in the worlds in which they live?

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